

# **[<sup>32</sup>P]2-iodo-*N*<sup>6</sup>-methyl-(*N*)-methanocarba-2'-deoxyadenosine-3',5'-bisphosphate ([<sup>32</sup>P]MRS2500), a novel radioligand for quantification of native P2Y<sub>1</sub> receptors**

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**1** Analysis of the P2Y family of nucleotide-activated G-protein-coupled receptors has been compromised by the lack of selective high-affinity, high-specific-radioactivity radioligands. We have pursued quantification of the P2Y<sub>1</sub> receptor through the development of a series of selective P2Y<sub>1</sub> receptor antagonists.

**2** Recently, we synthesized 2-iodo-*N*<sup>6</sup>-methyl-(*N*)-methanocarba-2'-deoxyadenosine 3',5'-bisphosphate (MRS2500), a selective, competitive antagonist that exhibits a *K*<sub>i</sub> of 0.8 nM in competition-binding assays with [<sup>3</sup>H]MRS2279. A 3'-monophosphate precursor molecule, MRS2608, was radiolabeled at the 5' position with <sup>32</sup>P using polynucleotide kinase and [<sup>γ</sup>-<sup>32</sup>P]ATP to yield [<sup>32</sup>P]MRS2500.

**3** [<sup>32</sup>P]MRS2500 bound selectively to Sf9 insect cell membranes expressing the human P2Y<sub>1</sub> receptor (Sf9-P2Y<sub>1</sub>), but did not detectably bind membranes expressing other P2Y receptors. P2Y<sub>1</sub> receptor binding to [<sup>32</sup>P]MRS2500 was saturable with a *K*<sub>D</sub> of 1.2 nM. Agonists and antagonists of the P2Y<sub>1</sub> receptor inhibited [<sup>32</sup>P]MRS2500 binding in Sf9-P2Y<sub>1</sub> membranes with values in agreement with those observed in functional assays of the P2Y<sub>1</sub> receptor.

**4** A high-affinity binding site for [<sup>32</sup>P]MRS2500 (*K*<sub>D</sub> = 0.33 nM) was identified in rat brain, which exhibited the pharmacological selectivity of the P2Y<sub>1</sub> receptor. Distribution of this binding site varied among rat tissues, with the highest amount of binding appearing in lung, liver, and brain. Among brain regions, distribution of the [<sup>32</sup>P]MRS2500 binding site varied by six-fold, with the highest and lowest amounts of sites detected in cerebellum and cortex, respectively.

**5** Taken together, these data illustrate the synthesis and characterization of a novel P2Y<sub>1</sub> receptor radioligand and its utility for examining P2Y<sub>1</sub> receptor expression in native mammalian tissues.

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**Abbreviations:** A3'MP, adenosine-3'-monophosphate; MRS2279, 2-chloro-*N*<sup>6</sup>-methyl-(*N*)-methanocarba-2'-deoxyadenosine-3',5'-bisphosphate; MRS2500, 2-iodo-*N*<sup>6</sup>-methyl-(*N*)-methanocarba-2'-deoxyadenosine 3',5'-bisphosphate

## **Introduction**

Extracellular nucleotides signal through two classes of membrane-bound receptors to mediate a multiplicity of intracellular responses. The P2X receptors are ligand-gated ion channels and are primarily activated by ATP. The P2Y receptors are seven-transmembrane-spanning G-protein-coupled receptors, and are activated by adenine and uridine nucleotides. The P2Y receptor family consists of eight members that can be subclassified based on selectivity of G protein coupling and sequence homology. P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, and P2Y<sub>11</sub> receptors couple to the G<sub>q</sub> class of guanine nucleotide-binding proteins, which signal downstream to trigger inositol lipid hydrolysis and subsequent mobilization

of intracellular calcium. The P2Y<sub>11</sub> receptor also couples to the G<sub>xs</sub> family of G proteins to stimulate adenylyl cyclase. The P2Y<sub>12</sub>, P2Y<sub>13</sub>, and P2Y<sub>14</sub> receptors exhibit high sequence homology and couple to the G<sub>xi</sub> family of G proteins, resulting in inhibition of adenylyl cyclase activity (Burnstock, 1996; Burnstock & Knight, 2004).

The P2Y<sub>1</sub> receptor is preferentially activated by ADP, while ATP is a weak partial agonist, and UTP and UDP are inactive (Schachter *et al.*, 1996; Leon *et al.*, 1997; Palmer *et al.*, 1998). This receptor plays an essential role in ADP-promoted platelet aggregation by triggering shape change and an initial, reversible phase of aggregation (Jantzen *et al.*, 1999). P2Y<sub>1</sub> receptor mRNA has been detected in numerous tissues (Janssens *et al.*, 1996; Leon *et al.*, 1996); however, a direct study of this receptor and its related physiology historically has been difficult due to the lack of a reliable radioligand-binding assay.

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We have developed a series of competitive antagonists that selectively inhibit P2Y<sub>1</sub> receptor-promoted signaling. Adenosine derivatives with phosphate groups at the 5' and 2' or 3' positions of the ribose ring were initially identified as selective, competitive antagonists (Boyer *et al.*, 1996). Structure-activity studies for adenosine bisphosphate derivatives substituted at various positions of the adenine and ribose rings along with molecular modeling and site-directed mutagenesis have led to the development of non-nucleotide-antagonists that are highly selective for the P2Y<sub>1</sub> receptor, exhibit low nanomolar potency for inhibiting downstream receptor signaling, and display limited susceptibility to metabolism by surface-localized nucleotide hydrolyzing enzymes (Jiang *et al.*, 1997; Boyer *et al.*, 1998; 2002; Camaioni *et al.*, 1998; Moro *et al.*, 1998; Nandanan *et al.*, 1999; 2000; Kim *et al.*, 2000; 2001; 2002). One of these molecules, 2-chloro-*N*<sup>6</sup>-methyl-(*N*)-methanocarba-2'-deoxyadenosine-3',5'-bisphosphate ([<sup>3</sup>H]MRS2279), was developed into an antagonist radioligand for the P2Y<sub>1</sub> receptor by a multi-step radio-synthetic scheme (Waldo *et al.*, 2002). While the development of a radioligand-binding assay using this molecule provides a reliable tool for quantification of recombinant P2Y<sub>1</sub> receptors and screening of new P2Y<sub>1</sub> receptor ligands, its low specific activity (89 Ci mmol<sup>-1</sup>) and intermediate affinity for the P2Y<sub>1</sub> receptor (*K*<sub>D</sub>: 8 nM) limit its general application for broadly quantifying P2Y<sub>1</sub> receptors in native mammalian tissues.

Recently, 2-iodo-*N*<sup>6</sup>-methyl-(*N*)-methanocarba-2'-deoxyadenosine-3',5'-bisphosphate (MRS2500) was synthesized as a competitive P2Y<sub>1</sub> receptor antagonist that inhibited [<sup>3</sup>H]MRS2279 binding with an affinity (*K*<sub>i</sub> = 0.79 nM) 10 times greater than MRS2279 (Kim *et al.*, 2003). We have chosen this molecule as a template to develop a higher-affinity, high-specific-radioactivity antagonist radioligand for the P2Y<sub>1</sub> receptor. MRS2500 was synthesized in radioactive form by the facile, single-step kinase-catalyzed phosphorylation of a precursor molecule to yield [<sup>32</sup>P]MRS2500 with a theoretical specific activity of 9120 Ci mmol<sup>-1</sup>. In this study, we describe the synthesis of this novel radioligand, the development of a high-specific-activity radioligand-binding assay for the P2Y<sub>1</sub> receptor, and the quantification of P2Y<sub>1</sub> receptors in various tissues of the adult rat.

## Methods

### Animals

Adult male Harlan Sprague-Dawley rats weighing 300–400 g were group housed and maintained on a 12:12 h light:dark cycle with access to food and water *ad libitum*. Animals were killed by decapitation by a trained laboratory animal technician. All procedures were carried out in accordance with the guidelines of the University of North Carolina Institutional Animal Care and Use Committee.

### Precursor for synthesis of MRS2500

The general synthetic approach for 2-iodo-*N*<sup>6</sup>-methyl-(*N*)-methanocarba-2'-deoxyadenosine-3'-monophosphate, MRS2608, a precursor of MRS2500, was described (Kim *et al.*, 2003). The detailed synthesis will be published separately.

### Enzymatic synthesis of [<sup>32</sup>P]MRS2500 from MRS2608

MRS2608 (50 nmol, 5 µl of a 10 mM solution in Tris, pH 7.5) was combined with 1.5 µl of 10× reaction buffer (500 mM Tris-HCl, 100 mM MgCl<sub>2</sub>, 50 mM dithiothreitol, 1 mM spermidine, and 1 mM EDTA, pH 7.5), 1 mCi of [<sup>32</sup>P]ATP (7 µl, 0.16 nmol, 150 mCi ml<sup>-1</sup>), and 2 µl (20 U) of 3'-phosphatase-free polynucleotide kinase. The sample was mixed by pipetting and the kinase-catalyzed reaction was incubated at 37°C for 1 h. The entire reaction volume was then injected onto a Luna 5µ C18(2) column (4.6 × 250 mm) at a flow rate of 1 ml min<sup>-1</sup> in a mobile phase of 5% acetonitrile/95% 0.1 M triethylammonium acetate (5% A/95% B). The column was washed for 30 min in 5% A/95% B to remove free [<sup>32</sup>P]ATP, and [<sup>32</sup>P]MRS2500 was eluted using a linear gradient of 5% A/95% B to 60% A/40% B over 50 min. [<sup>32</sup>P]MRS2500 eluted at 48 min, that is, 18 min after the start of the gradient (approximately 75% A/25% B). The precursor molecule, MRS2608, which was detected by UV (275 nm) eluted at 50 min. Fractions of 1 ml were collected during purification, and radioactivity in each fraction was quantified by liquid scintillation counting of a 5 µl aliquot of each fraction. [<sup>32</sup>P]MRS2500 has been purified by this procedure approximately 10 times, with a typical yield of approximately 20%. [<sup>32</sup>P]MRS2500 was stored at -20°C until use.

### P2Y<sub>1</sub> receptor expression in Sf9 insect cells

Sf9 insect cell membranes expressing recombinant P2Y receptors were prepared as described in detail previously (Waldo *et al.*, 2002). Briefly, recombinant baculoviruses encoding epitope-tagged constructs of the human P2Y<sub>1</sub>, P2Y<sub>2</sub>, or P2Y<sub>12</sub> receptors, or an avian P2Y receptor (Boyer *et al.*, 1997) were constructed using established protocols. Suspension cultures of Sf9 cells were infected with recombinant baculoviruses, and plasma membranes were prepared from uninfected (wild type) or infected cells after cell lysis and high-speed centrifugation. The membranes were frozen in aliquots at -80°C.

### Preparation of membranes from rat tissues

Adult male Harlan Sprague-Dawley rats were killed and organs were harvested and placed in 5 ml homogenization buffer (20 mM Hepes, pH 7.5, 145 mM NaCl, 5 mM MgCl<sub>2</sub>) per gram wet weight tissue. Whole organs or combined brain regions from groups of 2–6 rats were homogenized with a Polytron tissue disrupter for 45–60 s. Homogenized samples were centrifuged at 35,000 × *g* for 10 min. The resulting pellets were resuspended in 3 ml homogenization buffer per gram wet weight tissue and centrifugation was repeated two times. Final resuspensions were in homogenization buffer plus 5% glycerol and the samples were stored at -80°C. Protein concentrations were determined using the BCA protein assay.

### Radioligand-binding assay

Membranes were typically incubated with 0.1–0.25 nM [<sup>32</sup>P]MRS2500 in assay buffer (20 mM Hepes, 145 mM NaCl, 5 mM MgCl<sub>2</sub>, pH 7.5) in a 25 µl reaction volume in 12 × 75 mm<sup>2</sup> conical polypropylene tubes. Saturation-binding isotherms were generated at concentrations of [<sup>32</sup>P]MRS2500

ranging from 0.01 to 6 nM in a total volume of 20  $\mu\text{l}$ . Incubations were from 15 to 45 min in an ice-water bath and were terminated by the addition of 3.5 ml of ice-cold assay buffer followed by vacuum filtration over Whatman GF/A glass microfiber filters. The filters were washed with 7 ml ice cold assay buffer and radioactivity on each filter was quantified by liquid scintillation counting. Specific binding was defined as total  $[^{32}\text{P}]\text{MRS2500}$  bound minus binding occurring in the presence of 10 or 100  $\mu\text{M}$  MRS2179.

### Materials

3'-phosphatase-free polynucleotide kinase was from Roche Diagnostics Corp., Indianapolis, IN, U.S.A. MRS2179 was from Tocris-Cookson, Inc., Ellisville, MO, U.S.A.  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  was from Perkin-Elmer, Inc., Boston, MA, U.S.A. All other drugs were from Sigma-Aldrich Corp., St Louis, MO, U.S.A. The Luna 5  $\mu\text{m}$  C18(2) HPLC column was from Phenomenex, Inc., Torrance, CA, U.S.A.

### Data analysis

All experiments were carried out in duplicate or triplicate assays and were carried out at least three times or on samples from three individual animals. Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA, U.S.A.). Data are presented as the mean  $\pm$  s.e.m. from combined multiple experiments or in some cases as a data set from a typical experiment.

## Results

Structure-activity relationships for a series of synthetic adenine nucleotide analogs have led to the development of a class of non-nucleotide adenosine bisphosphate derivatives that selectively inhibit the  $\text{P2Y}_1$  receptor (Boyer *et al.*, 1998; Moro *et al.*, 1998; Nandanan *et al.*, 1999; Kim *et al.*, 2002; 2003). The replacement of the ribose ring of adenosine 3',5'-bisphosphate with a Northern-constrained cyclopentane structure and other modifications of the adenine base, including an  $N^6$ -methyl addition, have yielded molecules that are highly selective for  $\text{P2Y}_1$  over other  $\text{P2Y}$ ,  $\text{P2X}$ , and adenosine receptors. These non-nucleotide molecules are also presumed to circumvent the problem of nonspecific binding to the large number of other nucleotide-binding proteins present in mammalian cells. Recently, one of these molecules MRS2500, was found to interact with the  $\text{P2Y}_1$  receptor with subnanomolar affinity. This molecule was selected as the template for development of a high-specific-activity,  $^{32}\text{P}$ -labeled radioligand to quantify endogenous  $\text{P2Y}_1$  receptors in mammalian tissues.

### Synthesis of $[^{32}\text{P}]\text{MRS2500}$

MRS2500 inhibited binding of the  $\text{P2Y}_1$  receptor radioligand  $[^3\text{H}]\text{MRS2279}$  with a  $K_i$  value of 0.79 nM, and inhibited 2MeSADP-promoted inositol phosphate accumulation with a calculated  $K_B$  value of 1.74 nM (Kim *et al.*, 2003). A precursor to MRS2500 was generated with the goal of synthesizing a high-specific-activity radioligand. The precursor molecule, MRS2608, contains a phosphate group at the 3'-position and a hydroxyl group at the 5'-position, which potentially allows

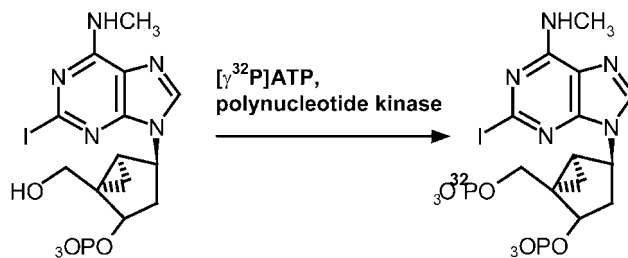
phosphorylation by polynucleotide kinase using  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  as the 5'-phosphate donor.

Reaction conditions for polynucleotide kinase-catalyzed radiophosphorylation were optimized using unlabeled ATP and adenosine-3'-monophosphate (A3'MP) as the phosphate acceptor. The extent of phosphorylation was quantified using ion exchange chromatography. Since polynucleotide kinase is known to exhibit small amounts of 3'-phosphatase activity, reactions were carried out with a mutant form of the enzyme containing a C-terminal deletion that results in ablation of its 3'-phosphatase activity (Wang & Shuman, 2002). Lack of 3'-phosphatase activity was confirmed using A3'MP as substrate (data not shown). A3'MP was stable in the presence of 3'-phosphatase-free polynucleotide kinase in the absence of ATP at 37°C for up to 24 h; incubation of A3'MP with unmodified polynucleotide kinase under identical conditions resulted in the appearance of a small amount of adenosine (data not shown).

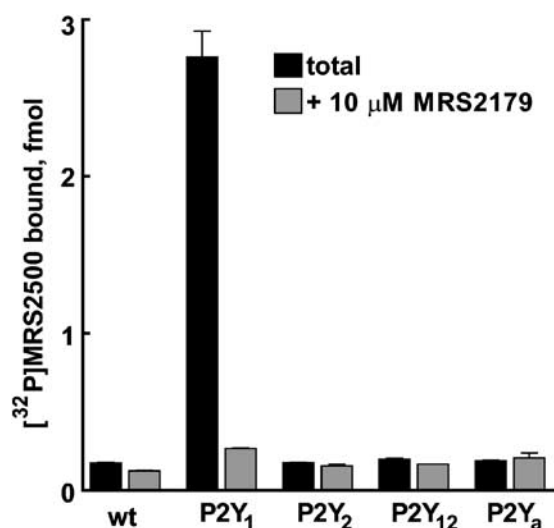
Reaction conditions that resulted in optimal phosphorylation of A3'MP were applied to  $^{32}\text{P}$ -phosphorylate MRS2608 to generate  $[^{32}\text{P}]\text{MRS2500}$  (Figure 1). Approximately 20% of the added  $[^{32}\text{P}]$  radioactivity was routinely recovered in a single peak that eluted from the reversed-phase column with a retention time of 48 min. The retention time of the radioactive product corresponded to the retention time of purified, unlabeled MRS2500 (Kim *et al.*, 2003) under the same mobile phase conditions. Contamination of purified  $[^{32}\text{P}]\text{MRS2500}$  with the precursor molecule MRS2608 was less than 1% in multiple purification procedures.

### Selectivity of $[^{32}\text{P}]\text{MRS2500}$ for the $\text{P2Y}_1$ receptor

To determine selectivity of the novel radioligand for the  $\text{P2Y}_1$  receptor,  $[^{32}\text{P}]\text{MRS2500}$  binding was evaluated in membranes from wild-type Sf9 (Sf9-wt) insect cells or Sf9 insect cells expressing human  $\text{P2Y}_1$  (Sf9-P2Y<sub>1</sub>),  $\text{P2Y}_2$  (Sf9-P2Y<sub>2</sub>), or  $\text{P2Y}_{12}$  (Sf9-P2Y<sub>12</sub>) receptors or the avian  $\text{P2Y}$  receptor (Sf9-P2Y<sub>a</sub>) (Boyer *et al.*, 1997). As shown in Figure 2,  $[^{32}\text{P}]\text{MRS2500}$  binding in Sf9-P2Y<sub>1</sub> membranes was 15-fold higher than binding observed in Sf9-wt membranes, and was inhibited by 90% in the presence of the  $\text{P2Y}_1$ -selective antagonist MRS2179 (10  $\mu\text{M}$ ). In contrast,  $[^{32}\text{P}]\text{MRS2500}$  binding in Sf9-P2Y<sub>2</sub>, Sf9-P2Y<sub>12</sub>, and Sf9-P2Y<sub>a</sub> membranes was essentially identical to that observed in Sf9-wt membranes and was not



**Figure 1** Synthesis of  $[^{32}\text{P}]\text{MRS2500}$ . MRS2608 (5  $\mu\text{l}$  of a 10 mM solution) was combined with 1.5  $\mu\text{l}$  of  $10 \times$  reaction buffer, 1 mCi of  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  (7  $\mu\text{l}$ , 0.16 nmol, 150 mCi  $\text{ml}^{-1}$ ), and 2  $\mu\text{l}$  (20 U) of 3'-phosphatase-free polynucleotide kinase. The sample was mixed by pipetting and incubated at 37°C for 1 h. The entire reaction volume was then injected onto a Luna 5  $\mu\text{m}$  C18(2) column for purification under mobile phase conditions as described in Methods.



**Figure 2**  $[^{32}\text{P}]\text{MRS2500}$  binding in Sf9 membranes expressing P2Y receptors. Wild-type Sf9 membranes or membranes expressing the human  $\text{P2Y}_1$ ,  $\text{P2Y}_2$ , or  $\text{P2Y}_{12}$  receptors or the avian  $\text{P2Y}_a$  receptor,  $\text{P2Y}_a$  ( $10\ \mu\text{g}$  each) were incubated with  $220\ \text{pM}$   $[^{32}\text{P}]\text{MRS2500}$  in the presence or absence of  $10\ \mu\text{M}$  MRS2179 to determine non-specific binding. Values are reported as total fmol  $[^{32}\text{P}]\text{MRS2500}$  bound  $\pm$  s.e.m. from a representative experiment ( $n=3$ ).

affected by MRS2179. These results indicate that  $[^{32}\text{P}]\text{MRS2500}$  binds specifically to  $\text{P2Y}_1$  receptors but not to other P2Y receptors in Sf9 membranes.

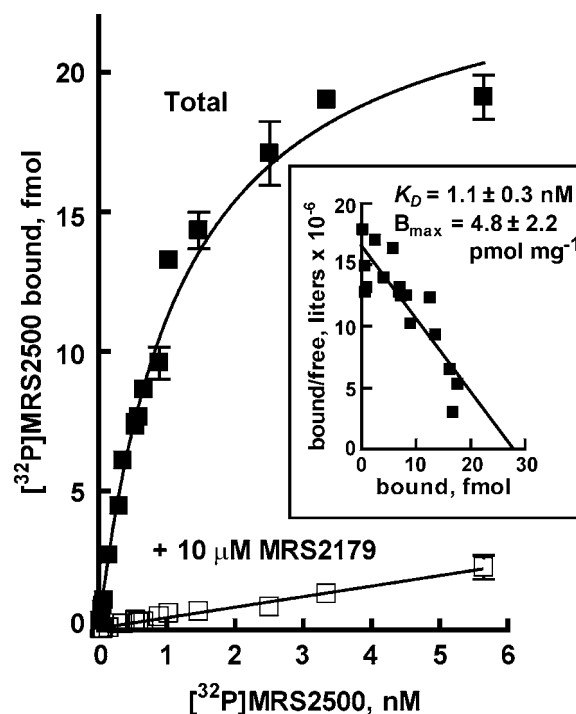
#### High-affinity binding of $[^{32}\text{P}]\text{MRS2500}$ to the $\text{P2Y}_1$ receptor

Optimal conditions for radioligand binding were determined in preliminary experiments. Specific binding occurring at  $4^\circ\text{C}$  was at least as great as that observed at room temperature (data not shown), and therefore all subsequent binding analyses were carried out at  $4^\circ\text{C}$ . Time-course experiments revealed rapid association of  $[^{32}\text{P}]\text{MRS2500}$  such that steady-state binding occurred within 2 min at  $4^\circ\text{C}$ . Prebound radioligand dissociated rapidly upon addition of a saturating concentration of MRS2179 ( $10\ \mu\text{M}$ ), with half of the bound radioligand dissociating within approximately 90 s.

Saturation-binding analysis was performed to determine the affinity of  $[^{32}\text{P}]\text{MRS2500}$  for the recombinant human  $\text{P2Y}_1$  receptor expressed in Sf9 membranes (Figure 3). Saturation-binding isotherms exhibited one-site binding kinetics with a  $K_D$  of  $1.1 \pm 0.35\ \text{nM}$  ( $n=3$ ) and an average  $B_{\text{max}}$  of  $4.8 \pm 2.2\ \text{pmol receptor mg}^{-1}\text{protein}$  in three experiments from a single membrane preparation.

#### Pharmacological selectivity of $[^{32}\text{P}]\text{MRS2500}$ binding

The capacity of several agonists and antagonists of the  $\text{P2Y}_1$  receptor and other P2Y receptors to compete with  $[^{32}\text{P}]\text{MRS2500}$  for binding was investigated in Sf9- $\text{P2Y}_1$  membranes. Owing to the high specific activity of  $[^{32}\text{P}]\text{MRS2500}$ , competition curves could be generated with minimal amounts of protein (250–500 ng), limiting the alteration of added nucleotides by membrane-bound nucleotide-metabolizing enzymes. Agonists known to bind to the  $\text{P2Y}_1$  receptor inhibited binding of  $[^{32}\text{P}]\text{MRS2500}$  in a concentra-



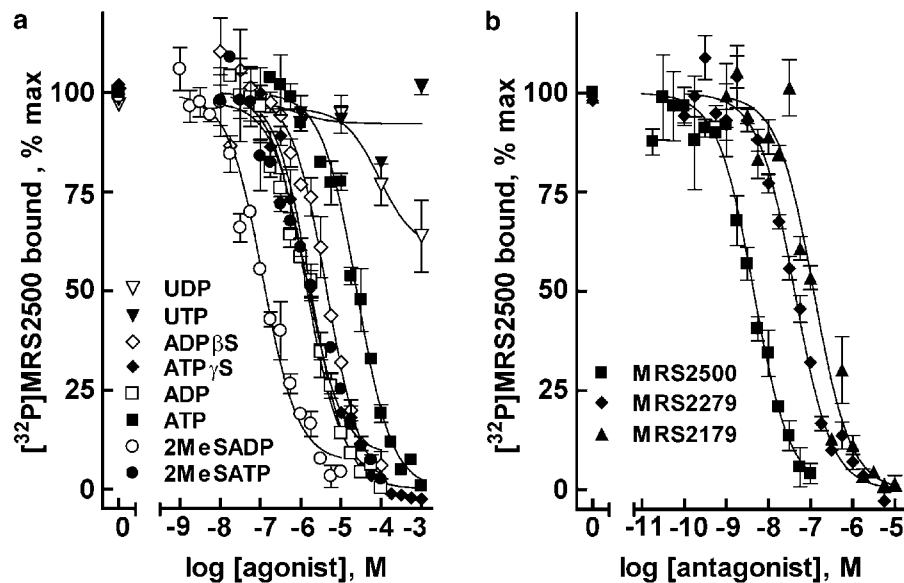
**Figure 3** Saturation-binding isotherm for  $[^{32}\text{P}]\text{MRS2500}$  binding to the human  $\text{P2Y}_1$  receptor. Sf9- $\text{P2Y}_1$  membranes ( $10\ \mu\text{g}$  per assay) were incubated for 45 min with the indicated concentrations of  $[^{32}\text{P}]\text{MRS2500}$  without or with the  $\text{P2Y}_1\text{R}$  selective antagonist MRS2179 ( $10\ \mu\text{M}$ ). Values are reported as total fmol  $[^{32}\text{P}]\text{MRS2500}$  bound  $\pm$  s.e.m. from a representative experiment ( $n=3$ ). Inset, Scatchard transformation of the data.

tion-dependent manner (Figure 4a). The rank order of potency observed was  $2\text{MeSADP} > 2\text{MeSATP} > \text{ADP} > \text{ATP}_\gamma\text{S} > \text{ADP}\beta\text{S} > \text{ATP}$ . This order was in agreement with the predicted potencies for the  $\text{P2Y}_1$  receptor based on previous observations of agonist-promoted  $\text{P2Y}_1$  receptor second-messenger signaling in cells continuously superfused with drug-containing medium (Palmer *et al.*, 1998). Moreover,  $K_i$  values (Table 1) were in excellent agreement with values determined in competition assays with  $[^3\text{H}]\text{MRS2279}$ , and the human  $\text{P2Y}_1$  receptor purified to homogeneity (Waldo & Harden, 2004). The  $\text{P2Y}_1$  receptor is known to bind adenine nucleotides specifically and is not activated by UTP or UDP; accordingly, uridine nucleotides did not compete with  $[^{32}\text{P}]\text{MRS2500}$  for binding to the  $\text{P2Y}_1$  receptor.

$\text{P2Y}_1$  receptor antagonists were also investigated for their capacity to compete with  $[^{32}\text{P}]\text{MRS2500}$  for binding to the  $\text{P2Y}_1$  receptor. MRS2179, MRS2279 and MRS2500 inhibited  $[^{32}\text{P}]\text{MRS2500}$  binding with  $K_i$  values in good agreement with  $K_B$  values determined for these same antagonists for inhibition of  $\text{P2Y}_1$  receptor-promoted second messenger signaling (Figure 4b, Table 1).

#### $[^{32}\text{P}]\text{MRS2500}$ binding in rat brain

One of the potential advantages of a high-specific-activity radioligand is high sensitivity for detection of receptors in native tissues. To determine the utility of  $[^{32}\text{P}]\text{MRS2500}$  for detection of  $\text{P2Y}_1$  receptors in native tissues, membranes were prepared from brains of adult male Sprague–Dawley rats. As



**Figure 4** Competition of  $\text{P2Y}_1$  receptor agonists and antagonists with  $[^{32}\text{P}]\text{MRS2500}$  for binding to  $\text{P2Y}_1$  receptor-expressing Sf9 membranes. (a) Sf9- $\text{P2Y}_1$  membranes (250 ng per assay) were incubated with 100 pM  $[^{32}\text{P}]\text{MRS2500}$  and the indicated concentrations of  $\text{P2Y}_1$  receptor agonists. (b) Sf9- $\text{P2Y}_1$  membranes (500 ng per assay) were incubated with 200 pM  $[^{32}\text{P}]\text{MRS2500}$  and the indicated concentrations of the  $\text{P2Y}_1$  receptor selective antagonists. Values are reported as % binding observed in the absence of competing ligand. Data shown are averages of triplicate samples  $\pm$  s.e.m. from a representative experiment.

**Table 1**  $K_i$  values for  $\text{P2Y}_1$  receptor agonists and antagonists in  $\text{P2Y}_1$  receptor-expressing Sf9 membranes

Agonist	n	$K_i$ ( $\mu\text{M}$ )	$K_B$ (nM)
2MeSADP	5	$0.05 \pm 0.01$	
2MeSATP	5	$0.49 \pm 0.10$	
ADP	4	$0.56 \pm 0.09$	
$\text{ATP}\gamma\text{S}$	3	$1.07 \pm 0.11$	
$\text{ADP}\beta\text{S}$	3	$2.30 \pm 0.60$	
ATP	3	$14.0 \pm 6.0$	
UTP	3	>1000	
UDP	3	>1000	
MRS2500	3	$2.35 \pm 0.48$	1.74 (Kim <i>et al.</i> , 2003)
MRS2279	5	$46.5 \pm 7.9$	8.91 (Boyer <i>et al.</i> , 2002)
MRS2179	3	$117 \pm 9$	102 (Boyer <i>et al.</i> , 1998)

Values reported are the average of three or more experiments  $\pm$  s.e.m.

shown in Figure 5a, saturation-binding analysis revealed binding of  $[^{32}\text{P}]\text{MRS2500}$  to a homogenous population of binding sites in rat brain with high affinity ( $K_D$ :  $0.33 \pm 0.02$  nM). An average  $B_{\text{max}}$  value of  $48.9 \pm 8.7$  fmol receptor  $\text{mg}^{-1}$  protein was determined ( $n=3$ ). To confirm the identity of this high affinity binding site as the rat  $\text{P2Y}_1$  receptor, pharmacological selectivity of  $\text{P2Y}_1$  receptor antagonists was examined. The  $\text{P2Y}_1$  selective antagonists, MRS2179, MRS2279, and MRS2500, competed for binding of  $[^{32}\text{P}]\text{MRS2500}$  in rat brain membranes with  $K_i$  values of  $1.97 \pm 0.74$ ,  $27.4 \pm 8.4$ , and  $267 \pm 72$ , respectively ( $n=3$ ). These values were in agreement with values obtained at the recombinant human  $\text{P2Y}_1$  receptor (Table 2). Taken together, these data demonstrate that  $[^{32}\text{P}]\text{MRS2500}$  is useful for quantification of  $\text{P2Y}_1$  receptors in adult rat brains. Preliminary studies revealed a large amount of breakdown of nucleotides by brain homogenates; therefore, we have not

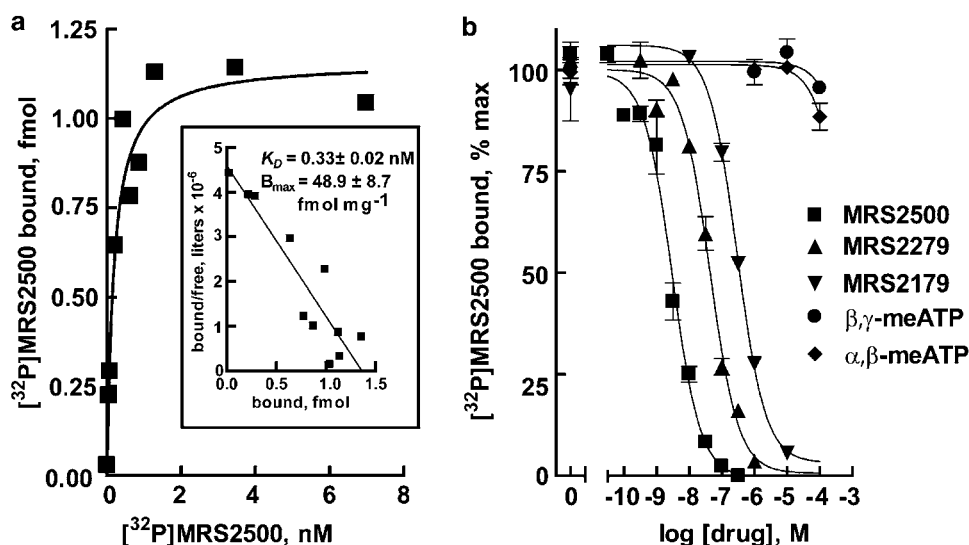
pursued agonist competition binding further in these studies of native  $\text{P2Y}_1$  receptors.

#### Tissue distribution of the rat $\text{P2Y}_1$ receptor

Having confirmed the utility of  $[^{32}\text{P}]\text{MRS2500}$  for labeling  $\text{P2Y}_1$  receptors in rat brain, we determined the relative density of  $\text{P2Y}_1$  receptors in a variety of rat tissues (Figure 6). Among tissues examined with a submaximal concentration of  $[^{32}\text{P}]\text{MRS2500}$  (4 nM), lung, liver, and brain exhibited the highest relative amounts of specific binding, with  $55 \pm 10$ ,  $31 \pm 3$ , and  $31 \pm 5$  fmol  $[^{32}\text{P}]\text{MRS2500}$  bound  $\text{mg}^{-1}$  protein, respectively. Heart, abdominal muscle, spleen, and stomach exhibited moderate receptor levels. Testes and kidney bound the least amounts of radioligand,  $6.5 \pm 2.4$  and  $2.7 \pm 1.7$  fmol  $[^{32}\text{P}]\text{MRS2500}$  bound  $\text{mg}^{-1}$  protein, respectively, and, in some cases, specific binding in these tissues was undetectable.

#### $\text{P2Y}_1$ receptor distribution in rat brain

$\text{P2Y}_1$  receptor mRNA is abundantly expressed in brain, and this receptor has been implicated in a number of neuronal physiologies, including regulation of neurotransmission, anxiolysis, and protection of astrocytes from oxidative stress-induced damage (Kittner *et al.*, 2003; Luthardt *et al.*, 2003; Shinzaki *et al.*, 2005). Saturation-binding analyses were performed in five major brain regions – cerebellum, cortex, midbrain, hypothalamus, and hippocampus. Among the brain regions examined, cerebellum exhibited the highest number of binding sites with a  $B_{\text{max}}$  value of  $112 \pm 17$  fmol  $[^{32}\text{P}]\text{MRS2500}$  bound per mg protein (Table 2). Midbrain, hypothalamus, and hippocampus displayed intermediate densities of binding sites, and cortex displayed the lowest number of binding sites with a  $B_{\text{max}}$  value of  $21.7 \pm 2.4$  fmol  $[^{32}\text{P}]\text{MRS2500}$  bound per mg



**Figure 5**  $[^{32}\text{P}]\text{MRS2500}$  binding in adult male rat brain. (a) Membranes prepared from adult male rat brain ( $30\ \mu\text{g}$  per assay) were incubated for 45 min with the indicated concentrations of  $[^{32}\text{P}]\text{MRS2500}$  without or with the  $\text{P2Y}_1$ R selective antagonist MRS2179 ( $100\ \mu\text{M}$ ). Inset, Scatchard transformation of the data. (b) Membranes from adult male rat brains ( $50\ \mu\text{g}$  per assay) were incubated with  $200\ \text{pM}$   $[^{32}\text{P}]\text{MRS2500}$  and the indicated concentrations of the indicated  $\text{P2Y}_1$  receptor antagonists. Values are reported as % binding observed in the absence of competing ligand. Data shown are averages of triplicate samples (a) or averages of triplicate samples  $\pm$  s.e.m. (b) from a representative experiment ( $n = 3$ ).

**Table 2**  $K_D$  and  $B_{\text{max}}$  values for  $[^{32}\text{P}]\text{MRS2500}$  binding in rat brain regions

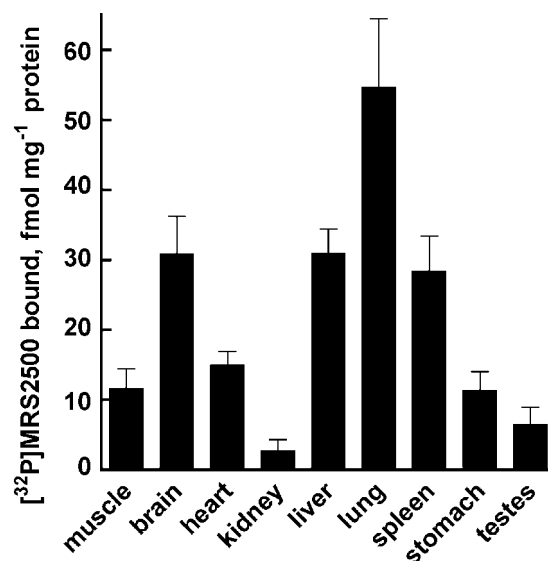
Region	$K_D$ (nM)	$B_{\text{max}}$ (fmol $\text{mg}^{-1}$ protein)
Whole brain	$0.33 \pm 0.02$	$48.9 \pm 8.7$
Cerebellum	$0.55 \pm 0.07$	$112 \pm 17$
Cortex	$0.47 \pm 0.06$	$21.7 \pm 2.4$
Hippocampus	$0.44 \pm 0.09$	$32.2 \pm 7.8$
Hypothalamus	$0.43 \pm 0.09$	$55.8 \pm 11.0$
Midbrain	$0.38 \pm 0.01$	$74.8 \pm 9.4$

Values reported are the average of three experiments  $\pm$  s.e.m.

protein. Thus,  $\text{P2Y}_1$  receptor expression varies by approximately six-fold among the major brain regions examined.

## Discussion

Study of the  $\text{P2Y}_1$  receptor has been significantly advanced by the development of selective pharmacological tools that directly target this signaling protein. In this report, we describe the synthesis and confirm the utility of  $[^{32}\text{P}]\text{MRS2500}$  as a novel high-affinity, high-specific-radioactivity antagonist radioligand for the  $\text{P2Y}_1$  receptor.  $[^{32}\text{P}]\text{MRS2500}$  binds selectively to the human  $\text{P2Y}_1$  receptor with a  $K_D$  of  $1.2\ \text{nM}$ . We have used this high-affinity radioligand to quantify  $\text{P2Y}_1$  receptors in a variety of rat tissues, and among the tissues examined, relative receptor levels were highest in lung, liver, and brain. We also examined receptor levels in several major brain regions and found a six-fold range of expression, with the highest and lowest densities of receptors found in the cerebellum and cortex, respectively. To our knowledge, this is the first unambiguous demonstration of a broadly useful high-specific-activity radioligand for a  $\text{P2Y}$  receptor natively expressed in mammalian tissues. Given the availability of the precursor,



**Figure 6**  $[^{32}\text{P}]\text{MRS2500}$  binding in adult rat tissues. Membranes prepared from various tissues of adult male Sprague–Dawley rats were incubated with  $4\ \text{nM}$   $[^{32}\text{P}]\text{MRS2500}$  in the presence or absence of MRS2179. Specific binding was normalized to protein amounts. Values are reported as fmol  $[^{32}\text{P}]\text{MRS2500}$  bound per mg protein. Data shown are averages of triplicate samples  $\pm$  s.e.m. from a representative experiment ( $n = 3$ ).

MRS2608, the preparative method is sufficiently simple to allow its convenient synthesis.

Development of selective  $\text{P2Y}_1$  receptor antagonists began with the identification of adenosine bisphosphate molecules as competitive antagonists. The presence of a  $5'$  phosphate group and an accompanying  $2'$  or  $3'$  phosphate group on the ribose moiety allowed recognition of these molecules by the  $\text{P2Y}_1$  receptor without receptor activation (Boyer *et al.*, 1996). Removal of the  $2'$  hydroxyl group of the ribose entity

eliminated interactions of adenosine-3',5'-bisphosphate analog with adenosine receptors, and addition of a methyl group at the  $N^6$  position conferred an increase in  $\text{P2Y}_1$  receptor-binding affinity (Boyer *et al.*, 1998). The discovery that interaction with the  $\text{P2Y}_1$  receptor was retained in bisphosphate analogs in which the ribose was replaced by acyclic or heterocyclic moieties (Kim *et al.*, 2000; 2001) was extended to the use of carbocyclic ribose-substituted heterocyclic bisphosphate analog constrained in either the Northern or Southern conformation by fusion of cyclopropane to a pseudosugar cyclopentane ring (Marquez *et al.*, 1996; Ezzitouni & Marquez, 1997). These bisphosphate methanocarba analogs retained affinity for the  $\text{P2Y}_1$  receptor, and  $N$ -methanocarba derivatives of  $\text{P2Y}_1$  receptor agonists and antagonists were more than 100-fold more potent than their corresponding  $S$ -isomers (Nandanan *et al.*, 2000; Kim *et al.*, 2002). Molecular modeling studies of the  $\text{P2Y}_1$  receptor based on the structure of rhodopsin confirmed that the Northern conformation was energetically favored by ligands docked in the putative  $\text{P2Y}_1$  receptor ligand recognition site (Nandanan *et al.*, 2000).

One goal of the development of non-nucleotide  $\text{P2Y}_1$  receptor antagonists was to reduce interaction of these molecules with other nucleotide-binding proteins, which hypothetically should be of value in our secondary goal of developing a useful radioligand for the  $\text{P2Y}_1$  receptor. Indeed, our studies of methanocarba analogs led to the synthesis of  $[^3\text{H}]\text{MRS2279}$ , and the binding of  $[^3\text{H}]\text{MRS2279}$  to membranes prepared from Sf9 insect cells expressing recombinant human  $\text{P2Y}_1$  receptors fit the pharmacological properties of the  $\text{P2Y}_1$  receptor (Waldo *et al.*, 2002). The  $[^3\text{H}]\text{MRS2279}$  radioligand-binding assay has allowed efficient screening of novel ligands for the  $\text{P2Y}_1$  receptor (Kim *et al.*, 2002; Waldo *et al.*, 2002) and has been applied to quantify the  $\text{P2Y}_1$  receptor during purification to homogeneity (Waldo & Harden, 2004). Although  $[^3\text{H}]\text{MRS2279}$  proved useful for quantification of  $\text{P2Y}_1$  receptors in human platelets (Waldo *et al.*, 2002), its relatively low specific activity ( $89\text{ Ci mmol}^{-1}$ ) has limited its use in other tissues in which the receptor is endogenously expressed. Thus, development of  $[^{32}\text{P}]\text{MRS2500}$ , which exhibits 10-fold higher affinity and 100-fold higher specific radioactivity than  $[^3\text{H}]\text{MRS2279}$ , represents an important step in ligand development for the unambiguous study of  $\text{P2Y}_1$  receptor-binding sites in mammalian tissues.

Previous work has investigated the tissue distribution of the rodent  $\text{P2Y}_1$  receptor using *in situ* hybridization techniques (Tokuyama *et al.*, 1995; Janssens *et al.*, 1996; Leon *et al.*, 1996; Moran-Jimenez & Matute, 2000). These studies suggest a broad expression pattern for the  $\text{P2Y}_1$  receptor among peripheral tissues and in rodent brain. Although *in situ* hybridization studies provide important insight into the relative distribution of this signaling protein, the relationship of mRNA to expressed functional receptors is unknown and is not likely to be constant. Antibodies that specifically recognize  $\text{P2Y}$  receptors would allow direct immunocytochemical quantification of receptor protein, but these tools also do not necessarily identify functional receptor-binding sites. Moreover, although antibodies against the  $\text{P2Y}_1$  receptor have been reported (Fong *et al.*, 2002; Yoshioka *et al.*, 2002; Franke *et al.*, 2003; Scheibler *et al.*, 2004), evidence for their selectivity is limited and their general reliability is uncertain.

The results described here illustrate that  $[^{32}\text{P}]\text{MRS2500}$  is a useful radioligand for quantification of functional  $\text{P2Y}_1$  receptor-binding sites across a wide range of mammalian tissues, and the remarkably high ratio of specific to nonspecific binding of this high-affinity, high-specific-activity radioligand allows reliable detection of binding sites to at least  $1\text{ fmol mg}^{-1}$  protein. Application of  $[^{32}\text{P}]\text{MRS2500}$  revealed a broad expression pattern for the functional receptor protein among peripheral tissues and rodent brain. Interestingly, this pattern is similar to that previously reported for messenger RNA (Tokuyama *et al.*, 1995; Janssens *et al.*, 1996; Leon *et al.*, 1996; Moran-Jimenez & Matute, 2000).

Tissue distribution data from our studies and other studies suggest potentially important physiological consequences of  $\text{P2Y}_1$  receptor signaling. The role of the  $\text{P2Y}_1$  receptor in ADP-promoted platelet aggregation is now well established (Gachet, 2001). However, its function remains largely undefined in the majority of tissues. Several studies have investigated the importance of  $\text{P2Y}_1$  receptor signaling in the central nervous system. ATP released from nerve terminals acts as an excitatory neurotransmitter through ionotropic  $\text{P2X}$  receptors (Cunha & Ribeiro, 2000). Roles for adenine nucleotides in other neural processes have been proposed, and potentially important consequences of signaling involving the  $\text{P2Y}_1$  receptor have been suggested. For example, activation of the  $\text{P2Y}_1$  receptor inhibits glutamate release, and  $\text{P2Y}_1$  receptor-mediated inhibition of NMDA receptor-promoted signaling occurs in prefrontal and parietal cortex (Luthardt *et al.*, 2003; Rodrigues *et al.*, 2005). Activation of the  $\text{P2Y}_1$  receptor also has been associated with anxiolysis, astrocyte protection, and oligodendrocyte proliferation and migration in rats (Kittner *et al.*, 2003; Agresti *et al.*, 2005; Shinozaki *et al.*, 2005).

Our work illustrates that  $[^{32}\text{P}]\text{MRS2500}$  can be utilized to quantify  $\text{P2Y}_1$  receptors in very small tissue samples, and the relatively high affinity and high specific radioactivity of this radioligand also make it a good candidate for detection of these receptors using autoradiographic techniques. Previous studies have claimed autoradiographic detection of the rat  $\text{P2Y}_1$  receptor using  $[x^{33}\text{P}]\text{dATP}$  or  $[^{35}\text{S}]\text{dATP}\alpha\text{S}$  as radioligands (Simon *et al.*, 1997; Fong *et al.*, 2002), but we have previously shown that the enormous amount of binding ( $10\text{--}50\text{ pmol mg}^{-1}$  protein) observed with these radioligands is nonspecific (Schachter & Harden, 1997). A  $^{33}\text{P}$ -labeled radioligand,  $[^{33}\text{P}]\text{MRS2179}$ , was used previously to quantify  $\text{P2Y}_1$  receptors in human platelets (Baurand *et al.*, 2001). We suspect that  $[^{33}\text{P}]\text{MRS2179}$  may not be a generally applicable radioligand since its affinity for the  $\text{P2Y}_1$  receptor is 100-fold lower affinity than the affinity of  $\text{MRS2500}$ . We have demonstrated here the high selectivity of  $[^{32}\text{P}]\text{MRS2500}$  for the  $\text{P2Y}_1$  receptor, and predict that this selectivity will allow for a more accurate analysis of brain  $\text{P2Y}_1$  receptor-binding sites.

The work described here demonstrates the development of a new molecular tool for quantification of the  $\text{P2Y}_1$  receptor with high sensitivity and illustrates that active  $\text{P2Y}_1$  receptor-binding sites are broadly distributed across rat tissues and brain. A reliable means for quantification of the  $\text{P2Y}_1$  receptor should lead to better understanding of the complex signaling and physiology associated with this important signaling protein.

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